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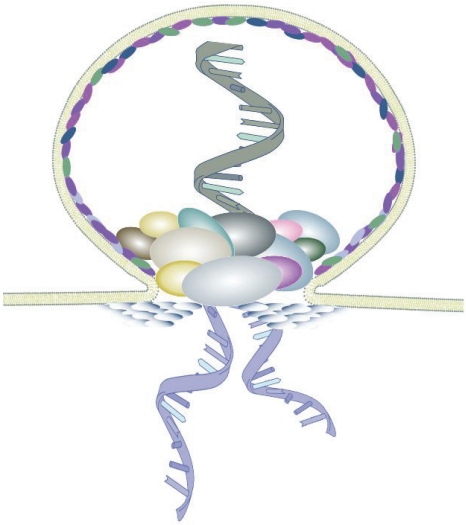
TANIA QUIRIN

REPLICASE PROTEINS UNDER SCRUTINY
TRANS-REPLICATION SYSTEMS TO DISSECT RNA VIRUS REPLICATION



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Replicase Proteins Under Scrutiny: *Trans*-Replication Systems to Dissect RNA Virus Replication

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“It is not the critic who counts; not the man who points out how the strong man stumbles, or where the doer of deeds could have done them better. The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood; who strives valiantly; who errs, who comes short again and again, because there is no effort without error and shortcoming; but who does actually strive to do the deeds; who knows great enthusiasms, the great devotions; who spends himself in a worthy cause; who at the best knows in the end the triumph of high achievement, and who at the worst, if he fails, at least fails while daring greatly, so that his place shall never be with those cold and timid souls who neither know victory nor defeat.”

— **Theodore Roosevelt**

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PUBLICATIONS

I. Versatile *Trans*-Replication Systems for Chikungunya Virus Allow Functional Analysis and Tagging of Every Replicase Protein.
PLoS One. 2016 Mar 10;11(3):e0151616.
Utt A, **Quirin T**, Saul S, Hellstrom K, Ahola T, Merits A.

II. Chikungunya Virus Infectivity, RNA Replication and Non-Structural Polyprotein Processing Depend on the nsP2 Protease's Active Site Cysteine Residue.
Sci Rep. 2016 Nov 15;6:37124.
Rausalu K, Utt A, **Quirin T**, Varghese FS, Žusinaite E, Das PK, Ahola T, Merits A.

III. The RNA Capping Enzyme Domain in Protein A is Essential for Flock House Virus Replication.
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I. TQ participated in the planning and design of the study. TQ tested the transcription and protein expression efficiency of the tagged and untagged T7-based replicase constructs. TQ performed and analysed the CLEM and immunofluorescence experiments for the paper. TQ wrote the corresponding parts of the paper.

II. TQ tested the transcription efficiency of the SFV and CHIKV protease mutants. TQ performed the Northern blot experiments and analysed the corresponding data for the paper. TQ participated in the writing process.

III. TQ designed all the experiments together with TA except the capture probe. TQ performed all the experiments except the capture probe and the construction of the protein A mutants. TQ performed the data analysis and wrote the paper together with TA, with suggestions and contributions from other authors.

ABSTRACT

My doctoral thesis examines the prerequisites of replication for three positive-strand RNA viruses, Chikungunya virus (CHIKV - alphavirus), Semliki Forest virus (SFV - alphavirus) and Flock House virus (FHV - nodavirus).

CHIKV is a mosquito-borne RNA virus that causes high fever, conspicuous rashes and unbearable joint pain. Semliki Forest virus (SFV) has been extensively studied as a model to comprehend the replication strategies of alphaviruses because of its low pathogenicity. A characteristic feature of alphavirus replication is the formation of membranous invaginations termed spherules, associated with the plasma membrane. Spherules act as genome factories as they are the sites of active viral replication and release nascent viral RNA strands into the cytoplasm through a bottleneck-like structure. We created a *trans*-replication system specific for CHIKV that would be flexible and presents no danger to the scientist. In this system, the viral replicase proteins are expressed from a DNA plasmid while the RNA template is produced from a second plasmid, in mammalian cells. This allowed for the study of viral replication without generating infectious particles. It also enabled the visualisation of spherules and labelling of all viral replicase proteins with fluorescent or small immunological tags while preserving their function. Various mutations associated with noncytotoxic phenotypes were analysed and the results showed no correlation between the level of RNA replication and cytotoxicity. Moreover, the *trans*-replication system was used to elucidate that the cysteine residue of CHIKV nsP2 at position 478 is responsible for its protease activity and essential for replicase polyprotein processing. Trp479 of nsP2 also plays a vital role in RNA replication.

The insect nodavirus, FHV, verges upon the properties of a 'universal virus' as it can replicate in a wide range of hosts. Only the replicase protein A is required for its replication. An efficient FHV *trans*-replication system was established in mammalian cells. The outer surface of mitochondria displayed pouch-like invaginations with a 'neck' structure opening towards the cytoplasm. High-level synthesis of both genomic and subgenomic RNA was detected in vitro using mitochondrial pellets isolated from transfected cells. The newly synthesized RNA was found to be of positive polarity. This system was used to investigate the capping enzyme domain of protein A, both in cells and *in vitro*. Mutating the most conserved amino acids of the capping domain abolished or reduced viral RNA synthesis. Surprisingly, transfection of capped RNA template did not rescue the replication activity of the mutants. FHV and alphaviruses show evolutionarily intriguing similarities in their replication complexes and RNA capping enzymes.

The biological systems presented in this study offer valuable knowledge that could be exploited to understand the replication of other RNA viruses and also open up new avenues for the elucidation of key virus-host interactions.

ABBREVIATIONS

BHK – Baby hamster kidney
CFP – Cyan fluorescent protein
CHIKV – Chikungunya virus
CLEM – Correlative light electron microscopy
CMP – Crude mitochondrial pellets
CMV – Cytomegalovirus
COP5 – Py transformed mouse cells
CSE – Conserved sequence element
DNA – Deoxyribonucleic acid
EGFP – Enhanced green fluorescent protein
EMCV – Encephalomyocarditis virus
FHV – Flock house virus
Fluc – Firefly luciferase
G3BP – Ras-GAP SH3 domain-binding protein
Gluc – Gaussia luciferase
GT – Guanylyltransferase
HA – Influenza haemagglutinin
Huh7 – Human hepatoma cells
IRES – Internal ribosomal entry site
MOI – Multiplicity of infection
MT – Methyltransferase
NLS – Nuclear localization signal
nsPs – Non-structural proteins
ORF – Open reading frame
PARP1 – poly-ADP-ribose polymerase I
PCR – Polymerase chain reaction
Rluc – Renilla luciferase
RNA – Ribonucleic acid
RDRP – RNA-dependent RNA polymerase
Rpb1 – RNA polymerase II subunit B1
SFV – Semliki forest virus
SINV – Sindbis virus
TATase – Terminal adenosyl transferase ()
TNTase – Terminal nucleotidyl transferase ()
UTR – Untranslated region
VEEV – Venezuelan equine encephalitis virus

INTRODUCTION

1.1 Viruses

Viruses are minuscule hijackers of life. They are obligatory infectious agents that rely solely on the species they infect for their replication. The origin of viruses is contentious. To date, there are three main concepts as to where they might come from; the virus-first, the progressive and the regressive hypotheses[31, 103, 150]. The virus-first hypothesis assumes that the existence of viruses predates that of cellular organisms. From a 'primitive soup' of interacting and competing nucleic acids and proteins, viruses would have emerged as individual organisms[31]. Even if this speculation might explain why the genome replication strategies of the viral world is more diverse than that of the cellular world, it has been widely rejected for two reasons: 1) in an early RNA world of free molecules, protein synthesis would have been a major challenge and, 2) our understanding of contemporary viruses suggests that an intracellular stage would have been necessary[31]. The progressive hypothesis, also known as the escape hypothesis, suggests that genetic elements gained mobility and eventually were able to exit cells and enter others. This parasitic-driven premise proposed that bacteriophages were derived from bacterial genomes while eukaryotic viruses came from eukaryotes. However, the lack of homology with archaeal viruses remains unexplained.[103] The regressive (or reduction) hypothesis suggests that the ancestors of viruses were complex, self-sufficient and lived in symbiosis with other organisms. Over time, the symbiotic relationship turned parasitic due to the loss of essential genetic information until they were unable to replicate independently. This theory is questionable as no intermediate has been identified, although some argue that the Mimivirus is the missing link between a cell and a virus[106]. Each one of these hypotheses have serious drawbacks. Nonetheless, by one way or another, it is undeniable that viruses have played a critical role in evolutionary shifts.

1.2 Positive-strand RNA viruses

The genetic material of RNA viruses can be single-stranded or double-stranded; positive sense or negative sense. Positive sense (or plus-strand) RNA viruses possess genetic material that is ready for translation by the host cell upon virus entry. Although the diversity of these viruses is immense, they do share commonalities; plus-strand RNA viruses code for an RNA-dependent RNA polymerase (RdRp) and their replication occurs in 'organelles' or compartments built

from their host's membranous structures. The type of membrane favoured is different for each virus family. For example, picornaviruses prefer to replicate in double-membraned vesicles originating from the Golgi membranes whereas alphaviruses prefer the plasma membrane[48]. There are about 214 RNA viruses known to infect humans and more are discovered every year[151]. Most RNA viruses infecting humans have zoonotic origins meaning that they can infect other vertebrate hosts or were able to do so previously. While it is possible for some viruses to cross species barriers and become human-adapted viruses, this happens very rarely[80]. In this study, we focused on the families *Togaviridae*, specifically the genus alphavirus (Chikungunya virus – CHIKV and Semliki forest virus – SFV) and an alphanodavirus (Flock house virus – FHV). Out of these three, only CHIKV can cause serious illness in humans.

1.3 Alphaviruses

Alphaviruses are arthropod-borne and are usually transmitted to humans via mosquito vectors[11, 126, 134]. Alphaviruses have been found in all continents except Antarctica. There are about 30 species recorded so far and they are often referred to as Old World or New World viruses according to their geographical distribution and historical occurrence[108]. Some of them are listed in Table 1 and include the locations where strains have been isolated as well as their medical relevance. Most of the Old World alphaviruses of medical importance cause arthritis-like syndromes whereas the New World alphaviruses cause encephalitis. The Old World viruses have been typically discovered in Eurasian-African-Australasian regions whereas the New World alphaviruses were found in the Americas. This worldwide distribution of alphaviruses has been attributed to various factors. For example, Sindbis virus (SINV) isolates have a very wide global distribution and are believed to have been propagated by avian hosts. In fact, several strains of alphaviruses have been isolated in migratory birds and it appears that, once introduced in a new area, these viruses have been able to establish a niche, evolve and persist in the formerly unaffected region[134]. However, in the case of CHIKV, human travel has been the cause of dissemination of the virus to the Caribbean islands, southern Europe and the Americas at a very rapid rate in 2013[30, 138, 146]. Newly-introduced alphavirus cases sometimes result in explosive epidemics but also fail to establish a persistent endemic cycle in the region (example: Ross River virus from Australia to the South Pacific) thus, resulting in the virus to die out after infecting most of the population[134].

Table 1: Classification of alphaviruses[13-14, 65, 88, 90, 108, 122, 134, 145, 147].

Old World alphaviruses	**Geographical distribution**	**Symptoms in humans**
Barmah Forest virus (BFV)	Australia	Mild fever, rash, polyarthritis
CHIKV	North, South America, Africa, India, Indian Ocean islands, Europe	Fever, arthralgia, rash
Eilat virus (EILV)	Africa, Middle East	None
O'nyong'nyong (ONNV)	Africa	Fever, arthralgia, rash
Ross River virus (RRV)	Australia, Oceania	Fever, arthralgia, rash
Sagiyama virus (SAG)	Japan	None
SINV	Africa, Asia, Europe, Australia, Scandinavia	Mild fever, rash, muscle pain
SFV	Africa, Eurasia	Fever, myalgia, arthralgia
New World alphaviruses	**Geographical distribution**	**Symptoms in humans**
Eastern equine encephalitis virus (EEEV)	North, South America	Febrile illness, encephalitis
Everglades virus (EVE)	Florida	Fever, myalgia, pharyngitis
Highlands J virus (HJ)	Eastern USA	None
Mayaro (MAY)	South America	Acute febrile illness, arthralgia, myalgia, rash
Venezuelan equine encephalitis virus (VEEV)	Central, North, South America, Mexico	Febrile illness, encephalitis
Western equine encephalitis virus (WEEV)	North, South America	Febrile illness, encephalitis

1.3.1 Semliki Forest virus (SFV)

In 1942, SFV was isolated from *Aedes* (*Ae.*) mosquitoes caught in an uninhabited area of the Semliki Forest in Uganda (Bwamba County)[127]. The virus was found to be pathogenic to mice, rabbits and guinea pigs after subcutaneous injections or lethal following intracerebral inoculations. Several passages in mice and rabbits enhanced the virulence of SFV ultimately causing death. Rhesus monkeys developed mild fever for 2 days following subcutaneous inoculation with the virus but the symptoms did not persist. However, intracerebral inoculation of healthy rhesus monkeys was lethal[126]. The strains of SFV are usually avirulent for humans. So far, there has been only one reported fatal case of SFV encephalitis in a laboratory worker. Some variants of SFV in central Africa can cause severe headache, fever, myalgia and arthralgia[134].

1.3.2 Chikungunya virus (CHIKV)

In 1952, an outbreak affecting the Makonde people of Tanzania was reported. The symptoms were similar to Dengue fever. However, the pathogen isolated in that case was a novel arbovirus[81, 87]. It is very plausible that previous outbreaks associated with Dengue virus were in fact caused by CHIKV as early as the year 1779[125]. CHIKV causes high fever, acute myalgias, rash and chronic severe arthritis[81, 134]. The name Chikungunya was given due to the contorted posture the patients would adopt due to painful arthralgia. It is derived from the Makonde root verb *kungunyala* and means 'to walk bent over'[86].

Genotypes of CHIKV are classified based on the E1 gene namely, the Asian (A), East/Central/South African (ECSA) and West Africa (WA) genotypes[87, 125]. The current consensus is that all the CHIKV strains originated 500 years ago from a common ancestor in East Africa and the virus was introduced in Asia in the 1950s[148]. While these genotypes used to be confined to their location, cases of ECSA have been reported in Asia as well[125]. The CHIKV *trans*-replication system described in publication **I** was designed based on an ECSA strain isolated from Reunion island in 2006 (LR2006-OPY). ECSA is not always confined to its geolocation as it has been detected in West Africa. This means that the genotypes can sometimes overlap spatially[148].

In 2004, a CHIKV epidemic started in East Africa. By 2005, the disease had spread from Kenya to Mauritius, La Réunion and other islands in the Indian Ocean[148]. The strain isolated from La Réunion was of ECSA lineage and was

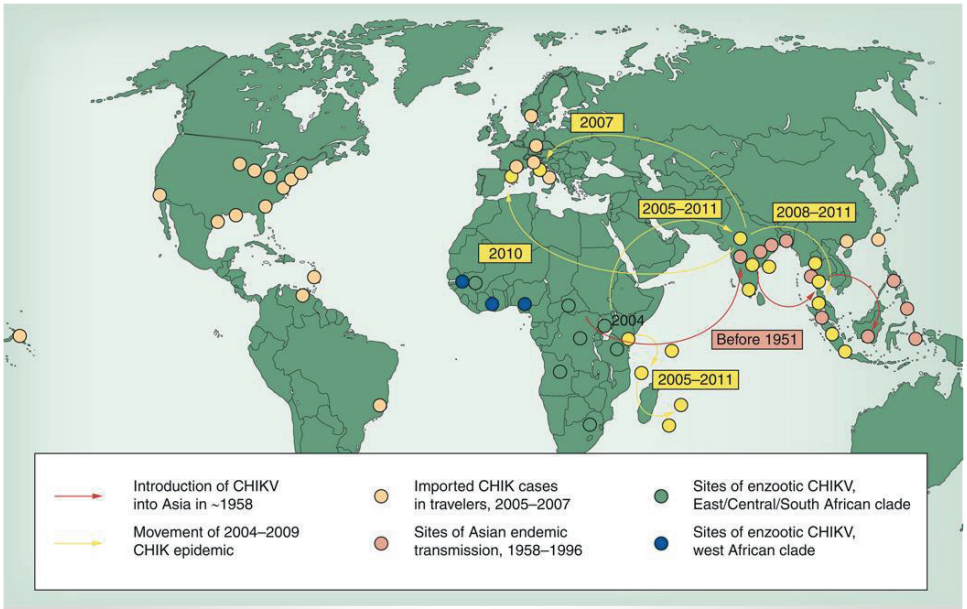


Figure 1: Worldwide distribution of CHIKV strains and movement of outbreaks (Reproduced from Ref [148], Copyright © 2019 Taylor & Francis; permission to reprint http://www.tandfonline.com).

found to have an A226V mutation in the E1 glycoprotein that conferred a considerable increase in infectivity and viral dissemination in *Ae. albopictus* but not *Ae. aegypti*, the usual host[139]. *Ae. albopictus* can survive winters in temperate climates in contrast to *Ae. aegypti*. Hence, this acquired advantage favours persistence of the virus in these regions where *Ae. aegypti* was scarce. In late 2005, epidemics ensued in India and from there, spread to southern Europe, southeast Asia and the Americas[148]. Then in 2009, another isolate was detected harbouring a L210Q mutation in the E2 glycoprotein. This mutation increased virus infectivity for *Ae. albopictus* although not as much as the A226V mutation[94]. All these events are depicted in Figure 1[148]. There were no current CHIKV outbreaks at the time this thesis was written

1.3.3 Structure of the virion and life cycle

Alphaviruses consist of an icosahedral nucleocapsid (T = 4) surrounded by a close-fitting envelope. The fenestrated nucleocapsid is composed of 240 copies of a capsid protein of about 30 kDa[55, 134]. It has been suggested that the numerous Arginine, Lysine and Proline residues at the N-terminus of the capsid protein protrude inward and interact electrostatically with the viral RNA within

the nucleocapsid. Enclosed in the nucleocapsid is a single-stranded RNA of positive polarity[134]. The envelope consists of a lipid bilayer of about 4.8 nm in thickness, derived from the host, in which 240 copies of E1 and E2 viral proteins are embedded. The envelope is rich in cholesterol and sphingolipids[55, 134]. E1 and E2 proteins are glycosylated, about 50 kDa each, and have membrane-spanning anchors in their C-terminal regions[134]. Together, E1 and E2 form homodimers and heterodimers, ultimately interlaced to form trimeric spikes on the surface of the virion[134]. E1 mostly stay at the base of the spikes and form a lattice on the surface of the virion. The N-terminus of E2 contains a receptor attachment site protruding externally in a leaf-like structure at the top of the spike whereas the C-terminus of E2 interacts with the nucleocapsid[55].

Upon infection of a mammalian cell, the virus attaches to the host's membrane receptor MXRA8 mostly via the protein E2[155]. The virion is then endocytosed in a clathrin-dependent manner[55]. In the endosome, ensuing events include a series of conformational changes as E1-E2 heterodimers disassemble to form E1 homotrimers and E2 monomers[55, 134]. This destabilisation occurs due to acidification as the endosome matures, consequently revealing the distal loop of E1. The viral bilayer fuses with the endosomal membrane and the nucleocapsid is released in the cytoplasm. The presence of cholesterol in the membrane is vital for this process to occur[55].

Next, the nucleocapsid disassembles in the cytoplasm, rendering the viral RNA available for replication to begin. Several hypotheses exist as to how this process occurs. It has been suggested that ribosomes facilitate the nucleocapsid uncoating process[149]. Alternatively, based on experiments involving *Ae. albopictus* cells, Lanzrein *et al* proposed that endosome acidification causes an amphiphilic portion of E1 to fold back into the membrane, creating a pore by interacting with other E1 subunits. The pores allow protons to freely enter the nucleocapsid and initiate uncoating[66]. Furthermore, endosomes undergoing acidification also undergo active Cl^- accumulation[129]. Considering that the nucleocapsid is destabilised by high salt concentration[19], it is likely that the release of viral RNA is a process that is initiated by endosomal maturation.

In the cytoplasm, the newly released viral RNA is translated by the host's cellular machinery and replicated. Alphavirus replication is explained in more detail in section 1.3.4. Briefly, the first two-thirds of the viral genome is translated into a polyprotein that is cleaved into non-structural proteins (nsPs) also referred to as replicase proteins. The nsPs form a complex that recruit and make several copies of the viral genomic RNA[134]. The complex also generates a smaller 26S

RNA from a subgenomic promoter within the viral genome that is translated into a structural polyprotein and cleaved into the capsid, E3, pE2 (precursor for E2 protein), 6K and E1[55].

Conserved sequences in the viral genome act as packaging signals[55]. A smaller protein, 6K, is also incorporated in the virion in smaller amounts than the other structural proteins (about one 6K molecule for every ten E1-E2 heterodimer)[55, 72]. 6K plays an important role in proper folding of E1-E2 heterodimers, affecting spike formation and egress[78]. Moreover, 6K was suggested to affect the selection of lipids to be used in virus assembly and facilitates the mechanisms involving membrane curvature in the budding process[72]. The absence of 6K appears to affect egress to a greater extent in mosquito cells compared to mammalian cells[72]. Assembly of viral particles is mediated by electrostatic interactions which also ensure the stability of the virion[134].

1.3.4 Viral genome and replication

The nsP amino acid (aa) sequences of CHIKV and SFV are 71% identical and therefore, the current understanding of CHIKV replication is partly derived from that of SFV replication[97]. The genome is a single-strand of RNA about 12 kb in length. The 5' end is capped and the 3' end is polyadenylated[60]. There are two open reading frames (ORFs) in the genome from which a non-structural polyprotein precursor and a structural polyprotein precursor are translated. CHIKV genome replication is portrayed in Figure 2. This process is active only during the first 6 – 8 hours of infection before the cellular translational machinery is shut down[119]. The non-structural polyprotein precursor can be expressed as P123 and P1234 due to the presence of an opal termination codon at the C-terminus of nsP3 and translational readthrough[134]. In some CHIKV isolates, such as the LR2006-OPY (ECSA) strain, the opal codon was found to be replaced by an arginine codon and therefore, this strain only expresses P1234 as the non-structural polyprotein precursor[119]. Next, P1234 is cleaved into individual nsPs and the RNA genome is recruited to the plasma membrane by nsP1. It is likely that the initiation of polyprotein processing (cleavage) precedes RNA recruitment although the exact order of events is unknown. The protease domain of nsP2 is responsible for polyprotein processing and the order of cleavages is based on the conformational changes the polyprotein undergoes during processing, the spatial rearrangement and the availability of the cleavage sites[74-75]. From P1234, nsP4 is cleaved first. Together, P123, nsP4 and the viral RNA form an early replicase complex. The early replicase complex launches the synthesis of an RNA strand

of negative polarity (minus-strand), complementary to the viral genome and hence, forms a dsRNA intermediate[67]. Concurrently, the plasma membrane bends, morphs and swells into a balloon-like structure termed spherule with an aperture facing the cytoplasm. In the spherule, further polyprotein processing occurs as nsP1 is cleaved next (in *cis* cleavage)[143]. The nsP1+P23+nsP4 complex is short-lived and rarely detected because of the very rapid subsequent step: cleavage of P23 into nsP2 and nsP3 (in *trans* cleavage)[143]. By the time the polyprotein has been cleaved to all the individual nsPs, minus-strand production is most likely completed. nsPs1-4 form a late replicase complex and use the minus-strand for

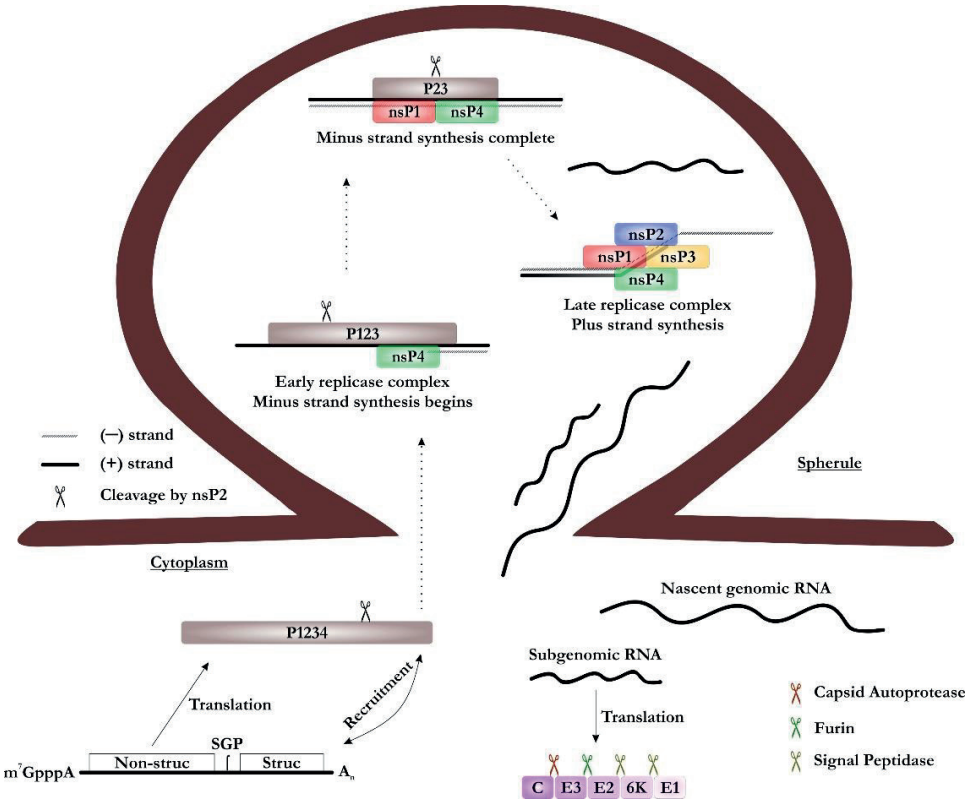


Figure 2: Polyprotein processing and viral RNA synthesis during CHIKV replication. P1234 is cleaved into the distinct nsPs while the RNA is recruited to generate a minus-strand, which is in turn used for the production of plus-strands. The replication complex is membrane-bound but its stoichiometry and its localisation within the spherule are unknown. The scissor icons represent the cleavage sites, SGP means subgenomic promoter, solid lines are plus-strands and dotted lines are minus-strands.

the generation of plus-strands (nascent genomic RNA) to be packaged in virions or to be recruited for the formation of new spherules. The late replicase complex also produces a smaller RNA, the subgenomic RNA (SgRNA). SgRNA exits the spherules to be translated by the cellular translational machinery as a structural polyprotein precursor to be further cleaved to C, E3, E2, 6K and E1 involved in the packaging and assembly of virions. Interestingly, it was found for SINV and SFV that, in the absence of the capsid protein, the viral genome can exit cells in the form of infectious microvesicles coated with spike proteins[111-112].

Cleavage events and formation of the replication complex are very dynamic events. It is likely that the replication complex undergoes several conformational changes when it switches from the plus-strand to using the minus-strand as the template. nsP2 is thought to assist in this process as the final cleavage events might inactivate minus-strand synthesis and plus-strand synthesis is initiated as the replication complex reassembles[117]. Whether the nsPs exist in an equimolar fashion is still unknown and so is the influence of host factors present in the spherule.

1.3.5 Replicase protein nsP1

As previously mentioned, the prerequisites for replication of the viral genome are the viral genomic RNA and the replicase proteins (Figure 3). However, nsPs also have other roles and have been found in cellular locations other than spherules. An amphipathic helix in the middle of nsP1 and palmitoylation are responsible for the anchoring of the replication complex to the plasma membrane[114, 133]. A study involving SFV polyproteins proved that nsP1 alone is responsible for the membrane targeting of the replication complex. In this study by Salonen *et al*, a cysteine residue at position 478 in the protease catalytic site of SFV nsP2 (further denoted as ^{CA}) was mutated to alanine to create uncleavable versions of SFV polyproteins. P12^{CA}3 and P12^{CA} were associated with the plasma membrane while P2^{CA}3 and P34 were associated with cytoplasmic areas of the cell[116].

In addition to its ability to act as a membrane anchor, nsP1 exhibits enzymatic features of methyltransferases (MT) and guanylyltransferases (GT). A histidine residue at position 38 in SFV nsP1 is vital for GT activity[4]. nsP1 is responsible for the 5' capping of the viral RNA genome, an important feature for alphavirus gene expression and conferring protection from targeted degradation. A capping reaction involves the methylation of GTP by nsP1, forming a covalent ^{7me}GMP+nsP1 complex, followed by the transfer of the ^{7me}GMP to the newly-

synthesised RNA genomic and SgRNA via the GT activity of nsP1. This results in a ^{7me}GpppA cap structure added to the 5' of the viral RNAs[2]. In the case of SINV, it was found that not all RNA strands are capped and the ratio of capped to non-capped viral RNAs depends on the host (mammalian cells vs mosquito cells). Moreover, the presence of non-capped viral genomic and SgRNA activated the host's innate immune system via the production of type I interferon[128]. SINV nsP1 remains functional in the absence of lipids, showing that membrane association is not vital for its enzymatic functions in contrast to SFV nsP1, thus showing a variation in the requirements for enzymatic activity among alphaviruses[114].

1.3.6 Replicase protein nsP2

nsP2 acts as a protease and helicase. The nucleoside triphosphatase (NTPase) and RNA-dependent 5'-triphosphatase enzymatic regions are positioned at the N-terminus whereas the proteolytic region is at the C-terminus of nsP2[140]. nsP2 is involved in polyprotein processing indicating that it is responsible for the cleavage of the polyprotein precursor into individual nsPs. This protease domain is essential for viral replication. In SINV, cleavage occurs in *trans* although the 1/2 and 3/4 sites can also be cleaved in *cis*. Moreover, the processing state of the polyprotein in which nsP2 is found determines the efficiency of protease activity at the nsP junctions[23, 114, 143]. For SFV, nsP2 has a high affinity for the 3/4 site[75, 144].

Due to aa sequence similarity between conserved sequences of *E.* coli helicases and the N-terminus of nsP2, it has been suggested that nsP2 is involved in duplex unwinding during RNA replication[43]. It was found that the aa residues 166– 630 of CHIKV nsP2 lack RNA helicase activity[58]. The lysine residue in the helicase region of nsP2 at position 192 is very conserved. The K192N mutation results in reduced infectivity but the progeny virions are capable of reversion[109]. The helicase properties of SFV nsP2 has been found to be dependent on Mg^{2+} and ATP concentrations[42]. During viral replication, nsP4 synthesises new RNA strands in a 5' to 3' fashion while nsP2 unwinds RNA in the same direction. Therefore, coordination and regulation of these two processes is critical[21].

When it is not part of the replication complex, CHIKV nsP2 localises in the nucleus where it can block the Jak-Stat signalling pathway and prevents transcription of interferon-stimulated genes[35]. Transcriptional shutoff is lethal for the cell. Furthermore, nsP2 mediates the degradation of Rpb1, the catalytic subunit

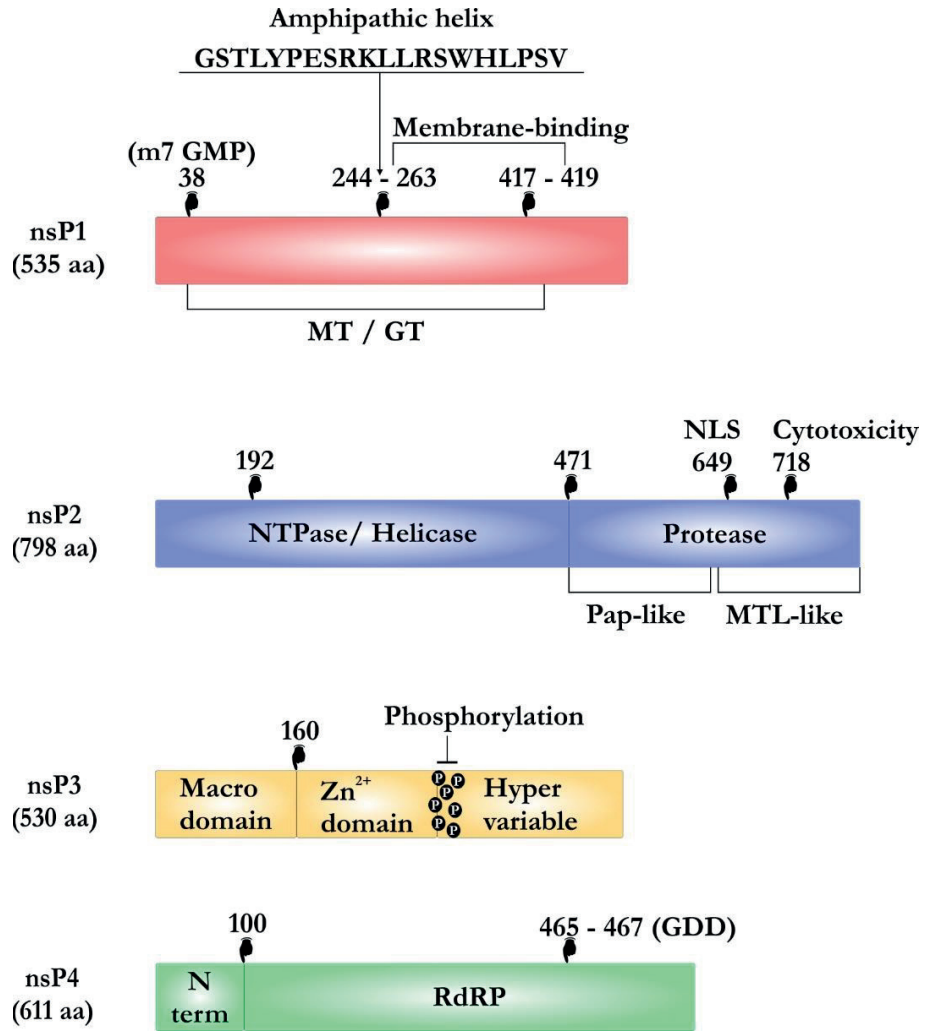


Figure 3: Schematic view of CHIKV nsPs. Amino acid residues of significance for the life cycle of the virus are shown (numbers).

of RNA polymerase II, by inducing ubiquitination and consequently causes disassembly of the RNA polymerase II complex. This leads to the termination of transcription and degradation via cellular pathways. The C-terminus and NTPase activity of nsP2 seem to be the culprits probably through the strong binding of nsP2 to DNA and locking it in a conformation whereby RNA polymerase II is unable to proceed[5]. The cytopathic effects seen in cells during alphavirus infections varies. For example, CHIKV nsP2 is not as efficient at shutting down the host's translation machinery compared to SINV nsP2[6]. The proline residue at

position 718 in CHIKV nsP2 is responsible for the cytotoxicity of nsP2 and substitution to glycine renders the protein less cytotoxic[135]. In SINV, the corresponding proline 726 mutation in the MT-like domain reduces cytotoxic effects and decrease viral replication[39]. Moreover, the nsP2 of New World viruses, such as VEEV, is rather harmless to the cell and is seen exclusively in the cytoplasm[62]. The VEEV capsid shuttles between the cytoplasm and the nucleus, inducing cytotoxicity by blocking nuclear transport via the CRM1 and importin α/β1 nuclear transport proteins[38, 76]. When the nuclear localisation signal (NLS) at position 649 of CHIKV nsP2 is altered, nuclear transport does not occur. While this is true for the WA CHIKV strain, the same mutation in the ECSA genotype does not have the same consequence[140]. Newly-synthesised RNA strands (both genomic and subgenomic) are processed to a 5' diphosphate moiety by the NTPase of nsP2 prior to capping[142].

1.3.7 Replicase protein nsP3

nsP3 is composed of a macrodomain, a zinc-binding domain and a hypervariable domain[140]. Macrodomains are very conserved across alphaviruses and some other unrelated viruses such as coronaviruses. Macrodomains bind to mono-ADP-ribose or poly-ADP-ribose and selectively remove ADP-ribose from aspartate and glutamate but not lysine residues[77, 79]. This feature appears to play an important role in the virus' attempt to fight against antiviral responses. Poly-ADP-ribose synthesis is enhanced by the nuclear polymerase PARP-1 due to inflammation and stress induced during alphavirus infection. The result is a decline in the level of ATP and NAD in the cell, and the release of an apoptosis-inducing factor[89]. Moreover, tampering with the CHIKV macrodomain either attenuates the virus or impairs replication completely[79]. Analysis of the crystal structure of CHIKV macrodomain revealed that the D10 residue is involved in the recognition of adenines thus rendering the macrodomain capable of binding to RNA[77].

Analysis of the Zinc-binding domain (also known as alphavirus unique domain – AUD) of SINV nsP3 showed that it contains a unique zinc-binding fold consisting of four cysteine residues coordinating one zinc molecule altogether having putative RNA binding capabilities[121]. In CHIKV, mutating the cysteine residues 262 and 264 to alanine renders the virus unable to replicate in rodent, mosquito and human cell lines. Moreover, mutations V260A/P261A close to the zinc-binding cysteines also abolish CHIKV viral replication either by preventing binding to important host factors or because of the change in the structural conformation of the protein[37].

The hypervariable domain possesses a hyperphosphorylated region and a proline rich region at the C-terminus which is believed to interact with several cellular host factors[44]. The proline rich region is the site for the binding of Src-homology (SH3) of amphiphysin-1 and amphiphysin-2 to nsP3[93]. Amphiphysin proteins have the ability to cause membrane curvature and thus could be recruited by the replication complex to assist in spherule formation[45, 47]. When it is not in the replication complex, nsP3 localises in large cytoplasmic aggregates. This feature of nsP3 is independent of viral replication as the protein alone has been detected in these foci[44]. During active CHIKV replication, nsP3 binds to the host protein Ras-GTPase-activating protein G3BP via two FGDF motifs in its sequence[99]. G3BP is an RNA-binding protein expressed in three isoforms that has an important role as a stress granule nucleating protein and aggregates are formed due to stress events occurring in cells[8, 137]. nsP3 recruits G3BP to its cytoplasmic aggregates, thus inhibiting stress granules formation[98].

Tampering with the N-terminal macrodomain does not affect this localisation but mutations in the hypervariable area of the C-terminal region gave rise to filamentous structures instead of foci. Moreover, deletions or point mutations in the SH3 domains, annihilates CHIKV replication. Association of nsP3 with G3BP is mediated via the SH3 domain[34]. Moreover, degradation of SFV nsP3 during infection is believed to play a role in regulating the levels of nsP4[44].

1.3.8 Replicase protein nsP4

nsP4 is the RNA-dependent RNA polymerase (RdRp) and contains a GDD motif necessary for its activity. nsP4 is usually expressed in smaller amounts compared to the other nsPs. The first reason for that is the presence of the opal codon readthrough as mentioned earlier. Secondly, in the case of SINV nsP4, the presence of a tyrosine residue at the N-terminus renders it highly unstable and subject to degradation by the ubiquitin-dependent N-end rule pathway[24]. Little is known about the N-terminus of CHIKV nsP4. However, previous studies with SINV nsP4 revealed that the N-terminus is highly flexible and mediates functional interactions between the nsPs[113]. A recombinant SINV nsP4 expressed as a N-terminal SUMO fusion protein revealed nsP4's ability to act as a terminal adenosyl transferase (TATase) and initiate *de novo* minus-strand synthesis *in vitro* in the presence of P123. Deletion of 97 aa at the N-terminus of nsP4 abolished RNA synthesis although the protein was still capable of terminal addition of nucleotides[110]. nsP4 being the most conserved protein between alphaviruses, it is very likely that CHIKV undergoes the same processes. Tight control

of nsP4 appears to be vital for alphavirus replication as the virus goes out of its way to limit its intracellular concentration to meet minimum requirements. Heat shock protein 90 (HSP-90) has been proposed to be of assistance in the stabilisation of nsP4 and chaperones the formation of the replication complex by directly interacting with nsP3 and nsP4[107]. Ribavirin is an RNA nucleoside analog that has a mutational effect on CHIKV nsP4 following multiple passages in mosquito and mammalian cells. The cysteine residue at position 483 was mutated to tyrosine. The result was an increase in replication fidelity and resistance to ribavirin and 5-fluorouracil as well[18]. Recently, a CHIKV *in vitro* replication assay was established by Albescu *et al* and revealed the synthesis of an RNA transcript, termed RNA II. The length corresponded to span from the 5' to the start of the subgenomic promoter. RNA II was also found in infected cells[7].

1.3.9 Spherule formation and cytopathic vesicles during alphavirus replication

Assembly of the early replication complex occurs in concert with spherule formation. CHIKV spherules emerge from the plasma membrane and have a neck-like opening to the cytoplasm. This opening is about 5-10 nm in diameter and guarantees that only the necessary components of replication such as nucleotides and other host factors, enter the spherule but also allows the exit of newly synthesised plus-strands of RNA[36]. As viral replication is initiated, a complementary minus-strand is generated from the first positive-sense RNA strand contained in the virus that entered the cell. Minus-strands are required for spherule formation but have not been detected in the cytoplasm[33, 49, 57, 131]. This suggests that the minus-strand most likely remains in the spherule, always acting as a template for RNA synthesis. Genomic and subgenomic RNAs are produced at the same time. Hence, it is possible that the replication complex is capable of synthesising multiple copies simultaneously. There is no evidence that the original plus-strand recruited exits the spherule after production of the minus-strand but it is logical to assume so; first because this strand would undoubtedly undergo the same 'spherule-exiting mechanism' as the newly synthesised plus-strands and secondly to free up space in an already crowded subcellular environment. Hence, this assumption implies that the replication of alphaviruses is semi-conservative. In a wildtype viral setting, spherules are usually about 60 nm in diameter. However, it has been shown with SFV that the size of the spherules depends on the length of the RNA[56].

Cytopathic vesicles (CPVs) are large cytopathic vacuoles resulting from the internalisation of a large number of spherules. This cellular event leads to the formation of a modified endosomal/lysosomal structure lined with spherules with the neck facing the exterior of the CPV[124, 132]. Internalisation of spherules happens frequently for SFV but to a lesser extent during SINV infection. CHIKV replication complexes stay mostly at the plasma membrane although internalisation and CPV formation have been described for this virus[102]. They are typically observed about 2 to 3 hours following alphavirus infection[46]. The appearance of the first CPVs depends on the multiplicity of infection (MOI). For example, CPVs can already be detected an hour after infection at a MOI of 200 whereas, at MOI 20, they are undetectable until 5 hours post infection[134].

1.4 Nodaviruses

The *Nodaviridae* family consists of viruses capable of replicating in a wide variety of invertebrates and vertebrates[104]. These viruses are further categorised as alphanodaviruses, infecting insects (examples: FHV, black beetle virus, Pariacoto virus) whereas betanodaviruses infect fish (examples: striped jack nervous necrosis virus, barfin flounder nervous necrosis virus). Betanodavirus outbreaks in China, Indonesia, Singapore and India affect the rearing of aquatic animals[154]. A third type of nodavirus infect shrimps and prawns (examples: Macrobrachium rosenbergii nodavirus and Penaeus vannamei nodavirus). These have been suggested to be classified as gammanodaviruses[92].

1.4.1 Flock house virus (FHV)

FHV is a non-enveloped, icosahedral virus that was first isolated from grass grubs (Costelytra zealandica) in New Zealand[120]. It is a unique virus because, although it only infects insects, it can replicate in several species once its genome is introduced into cells. For instance, FHV has been seen to replicate well in the yeast Saccharomyces Cerevisiae[104], Drosophila cells[15, 105], Caenorhabditis elegans[73], in planta[9, 157] and mammalian cells[52]. Recently, FHV has been suggested to be a prime candidate for the engineering of a virus-based RNAi delivery system and targeted gene silencing in insects[136].

1.4.2 FHV Viral replication and protein A

The FHV genome, as shown in Figure 4, is capped and segmented, consisting of RNA1 and RNA2. RNA1 encodes the viral replicase protein A and two smaller accessory proteins B1 and B2. Protein A possesses a membrane binding domain, self-interacts and is the RdRP[26]. Intact mitochondrial membranes are

required for FHV replication[152]. The RdRP domain of protein A contains a Gly-Asp-Asp (GDD) sequence that is essential for the catalytic activity of the polymerase. Protein A is the only protein required for the replication of FHV[141].

RNA2 codes for the coat precursor protein alpha which is cleaved to generate capsid proteins and participates in the regulation of RNA3 synthesis. Replication of RNA1 and RNA2 occur independently of each other. In the absence of RNA2, a shutoff of RNA1 replication was seen to occur approximately 3 days following transfection of *Drosophila* cells. Replication could be restored by transferring the intracellular RNA from the infected cells to fresh ones or by reactivating transcription[53]. Moreover, it appears that RNA3 can be replicated from minus-strands to plus-strands by the RdRp in the absence of RNA1[27]. A region within the sequence of protein A directs the synthesis of a SgRNA, RNA3, from which proteins B1 and B2 are translated. RNA3 controls the production of RNA1 and RNA2, a vital process for regulating the expression of the viral proteins and ensuring active replication[28, 70, 156]. The cellular decapping activators LSm1-7, Pat1, and Dhh1 coordinate the ratio of RNA1 to RNA3. Depletion of these decapping activators resulted in the accumulation of RNA3 in experiments performed in yeast cells by Giménez-Barcons *et al*[40].

B1 is encoded in the same reading frame as protein A whereas B2 is in a +1-reading frame compared to protein A[22]. The function of B1 is unknown. B2 is involved in immune responses in insect cells and transgenic plants, acting as an RNAi inhibitor[54, 69]. The first 73 aa of B2 binds dsRNA[16]. This ability has been exploited for the design of new immunological methods as this region can be used as a dsRNA-specific molecular probe *in vitro* and *in vivo*[84].

While it is not required for FHV replication, B2 increases its efficiency. In the absence of RNA2, a boost in RNA3 synthesis is observed in yeast cells[104]. A G-to-T nucleotide substitution at position 2721 at the start of RNA3 has a detrimental effect on SgRNA plus-strand synthesis but minus-strand synthesis is not affected. This shows that this position is critical for the good functioning of protein A[104]. Protein A is a 998 aa long polypeptide and has a mitochondrial localisation signal at the N-terminus[82]. Protein A possesses a terminal nucleotidyl transferase (TNTase) capable of restoring the loss of nucleotides at the 3'-terminus of the RNA template. This TNTase activity permits the reinitiating of RNA synthesis by a *de novo* mechanism[153]. Moreover, multiple aa sequences in protein A are involved in protein-protein interactions. There is likely to be a binding competition between these various regions of protein A suggesting that the protein has the ability to form multimers[12]. Moreover, tampering with these regions

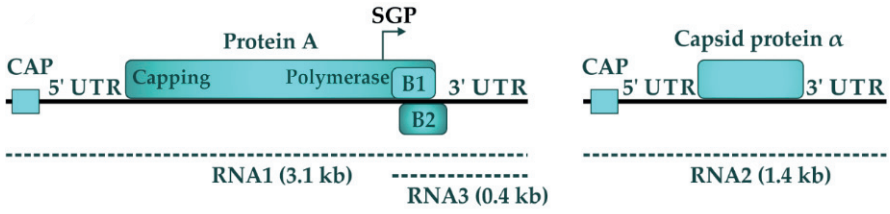


Figure 4: The FHV genome is bipartite and capped. Protein A is expressed from RNA1, proteins B1 and B2 are expressed from RNA3. Capsid protein α is expressed from RNA2. Dotted lines represent minus-strand, SGP is the subgenomic promoter (modified from **III**).

affects FHV replication. Thus, protein A self-interactions is crucial for viral genome replication[26].

1.4.3 Spherules emerge from mitochondrial outer membrane

Just like for alphaviruses, viral replication occurs in spherules but the composition is completely different as FHV spherules emerge from outer mitochondrial membranes[83]. These spherules eventually take all the mitochondrial intermembrane space and profoundly alter the morphology of mitochondria[64, 91, 100]. The neck of the spherule provides a connection to the cytoplasm and is approximately 10 nm in diameter[25]. It has been hypothesised that since protein A is a transmembrane protein, it probably lines the interior of the spherules. Based on the surface area of the spherule and the size of protein A, it was previously assumed that about 100-150 copies of protein A could fit in a densely packed spherule[63]. Recent studies involving the analysis of these spherules using cryo-electron tomography revealed interesting features of FHV replication. First, a crown-like structure was observed at the neck of the spherule, stabilising the invagination as it is strongly implanted in the membrane and, monitoring the influx and outflow like a gate. This structure had a cup-like main body and an outer ring of twelve projections most likely due to the multimerisation of protein A[29]. Spherules are very densely packed with both protein A and nucleic acids. Long filaments (nascent RNA strands) were seen exiting the spherule. Investigation of the size of the spherules and its correlation with the length of FHV's viral RNA proposes that some spherules contain between one and three copies of dsRNA and synthesis of several plus-strands may occur at the same time. Moreover, it was suggested that each viral RNA (RNA1, RNA2 and RNA3) is replicated in its own

spherule thus accounting for the wide range of different sizes of spherules observed[29]. Another function of the crown could be capping of the newly synthesised RNA strands as they are being pushed out of the spherules[29].

1.5 *Trans*-replication systems

Viral replication can occur either *in cis* (interactions within the same molecule) or *in trans* (interactions between different molecules). The replication of some positive-strand RNA viruses strictly occurs in *cis*. For example, the Kunjin virus (flavivirius) requires that some replicase proteins be translated *in cis* for replication to occur, thus being a limiting factor for the design of a *trans*-replication system for this virus[71]. Khromykh *et al* suggested that these cis-elements are important for the hydrophobic interactions occurring with membranes during viral replication[61]. It is possible that the presence of cis-acting elements facilitates the recognition of various cellular factors and viral RNAs. In the case of poliovirus, *trans*-replication can only occur *in vitro* but inefficiently[85, 95, 130]. For Tobacco mosaic virus, a molecular mechanism for *cis*-preferential replication has been proposed[59]. For other viruses including alphaviruses, replication can occur in *trans*.

Trans-replication systems have helped to elucidate pre-requisites of replicase complex assembly and RNA synthesis in alphaviruses[131]. In a complete *trans*-replication system, viral protein expression is uncoupled from RNA synthesis. Therefore, assembly of the replicase proteins, the importance of the RNA genome and spherule formation can be studied in detail. First, it was resolved for SINV that the expression of an uncleavable version of P123 and nsP4 possessing an N-terminal tyrosine residue, making up the early replicase complex, is capable of minus-strand synthesis. However, further cleavage of the polyprotein into nsPs is required for plus-strand synthesis[68]. In the case of SFV, it has recently been established that spherules can be formed in the absence of the RNA genome. In effect, the combination uncleaved P123+nsP4 produces abundant spherules[50]. This outcome suggests that perhaps all the host factors needed for replication and spherule formation to occur are already present when the early replicase is formed. Moreover, manipulations of the RNA genome in SFV *trans*-replication systems have revealed that the minus-strand formed during replication is not available for translation, re-affirming that it is well confined within the spherule, away from cytoplasmic events[49].

AIMS OF THE STUDY

The foremost aim of this study was to establish *trans*-replication systems specific for CHIKV and FHV. Other aims were:

1. To individually tag CHIKV replicase proteins with fluorescent markers or small immunological tags.
2. To investigate the protease activity of CHIKV nsP2.
3. To investigate the capping activity of FHV using cell-based and *in vitro* assays.
4. To compare the *trans*-replication systems of SFV and FHV.

MATERIALS & METHODS

The methodology, antibodies, molecular constructs and viruses used in this study are listed in the tables below. An asterisk denotes the author's contribution to the method. A more detailed description can be found in the original publications.

Table 2: Methods used in the study.

Method	Publication
Cell culture	
* BHK-21 (Hamster)	**I, II**
* BSR T7/5 (Hamster)	**I, II, III**
* U2OS (Human)	**I**
Huh7 (Human)	**I**
COP-5 fibroblasts (mouse)	**I**
DNA/RNA transfection	
* Lipofectamine	**I, II**
* PEI	**III**
Cell manipulation	
* Isolation of crude mitochondrial pellet	**III**
Virological replication assays	
* Luciferase measurements (intracellular)	**I, III**
* *In vitro* replication assay (extracellular)	**III**
Nucleic acid techniques	
* Molecular cloning	**I, II, III**
* DNA and RNA isolation & purification	**I, II, III**
* PCR	**I, II, III**
* Site-directed mutagenesis	**I, III**
* Northern blot	**I, III**
* *In vitro* RNA transcription	**I, III**
Capture probe	**III**
Microscopy	
* Confocal microscopy	**I, III**
* CLEM	**I, III**

Protein studies	
CD spectroscopy	**II**
Protease assay involving FRET (fluorescence resonance energy transfer)	**II**
Immunological methods	
* Western blot	**I, III**
Data analysis	
* ImageJ	**I, III**
* SkanIT varioskan software	**III**
* Odyssey infrared imaging system	**III**
* Typhoon Trio imager software	**III**
GraphPad prism	**I**

Table 3: Antibodies used in the study.

Primary antibodies	**Publication**
anti-CHIKV nsP1	**I, II**
anti-CHIKV nsP2	**I, II**
anti-CHIKV nsP3	**I, II**
anti-CHIKV nsP4	**I, II**
anti-Flag	**I**
anti-β-actin	**I, II**
anti-SDHA	**III**
anti-dsRNA (J2)	**I, II, III**
anti-HA	**III**
anti-Tom20	**III**

Table 4: Recombinant proteins CHIKV nsP2 (**Publication II only**).

wt His-nsP2
His-nsP2^{C478A}
His-nsP2$^{C478A+S482A}$
His-nsP2^{S482A}
His-nsP2^{W479A}

Table 5: Self-replicating replicons (producing both replicase proteins and RNA template).

CHIKV replicons	**Publication**
Repl-wt	**I**
Repl-1^{E}	**I**
Repl-2^{E}	**I**
Repl-3^{E}	**I**
Repl-4^{HF}	**I**
Repl-4^{SF}	**I, II**
Repl-1^{E}	**III**

Table 6: Viruses used in the study.

CHIKV Viruses	**Publ.**
ICRES-wt	**I**
ICRES-4^{SF}	**I**
ICRES-4^{HF}	**I**
pSP6-CHIKV^{C478A}	**II**
pSP6-CHIKV^{W479A}	**II**
pSP6-CHIKV^{S482A}	**II**
pSP6-CHIKV$^{C478A+S482A}$	**II**

Table 7: SFV T7 *trans*-replication systems.

SFV Replicases	Publication
P123Z4	**II**
P123Z4^{GAA}	**II**
P12^{CA}3Z4	**II**
P12^{WA}3Z4	**II**
P12^{SA}3Z4	**II**
P12^{CA+SA}3Z4	**II**
P123HA4	**III**
P123HA4_GAA	**III**
SFV Templates	**Publication**
Tmed	**III**
CFP_Stluc	**III**

Table 8: FHV T7 *trans*-replication systems (**Publication III only**).

FHV Replicases
P_HA
P_GAA
P_GAA_Vis
H93A
R100A
D141A
W215A
Δ2-35A
FHV Templates
T_Rluc
T_eGFP
FHV_T

Table 9: CHIKV *trans*-replication systems.

CHIKV Replicases			
	CMV	**T7**	**Publ.**
P1234	✓	✓	**I**
P1234-NAT	✓	✓	**I**
P1^{E}234-A	✓	✓	**I**
P1^{E}234-B	✓	✓	**I**
P1^{E}234-C	✓	✓	**I**
P1^{E}234-D	✓	✓	**I**
P12^{E}34-A	✓	✓	**I**
P12^{E}34-B	✓	✓	**I**
P12^{CA}34		✓	**II**
P12^{WA}34		✓	**II**
P12^{CA+SA}34		✓	**II**
P12^{SA}34		✓	**II**
P123^{E}4	✓	✓	**I**
P123^{E}4-GAA		✓	**I**
P1234^{E}	✓	✓	**I**
P1234^{SF}	✓	✓	**I**
P1234^{HF}	✓	✓	**I**
P1234^{HS}	✓	✓	**I**
P12^{EK}34	✓		**I**
P12^{KN}34	✓		**I**
P12^{5A}34	✓		**I**
P12^{FG}34	✓		**I**
P12^{EKPG}34	✓		**I**
P12^{5APG}34	✓		**I**
CHIKV Templates			
Fluc-Gluc	✓	✓	**I, II**
Rluc-Tom	✓	✓	**I, II**
Rluc-Tom-Vis		✓	**I**

RESULTS AND DISCUSSION

4.1 Ability of CHIKV to *trans*-replicate

A *trans*-replication system uncoupling translation and replication of viral RNA was established for CHIKV. The cellular RNA polymerase II promoter was used for the generation of CHIKV RNA templates (**I; Fig. 1B**) while viral replicase protein expression was driven by the CMV promoter (**I; Fig. 1A**). The first ORF encoded Firefly luciferase (Fluc) and hence, could be expressed in the absence of active CHIKV replicase by the cellular machinery. The second ORF encoded Gaussia luciferase (Gluc) and its expression correlated with the production of subgenomic RNAs. Thus, the expression of the second ORF in the templates of the *trans*-replication systems is the bona fide indicator of viral replication. We compared the Gluc activity of a CHIKV replicase plasmid, having native codon usage (ECSA genotype – LR2006 OPY1), to a replicase plasmid optimised for human codon usage, in BSR T7/5 cells, and observed a strong boost in viral replication from the latter (**I; Fig. 1C**). In order to broaden the application, five cell lines were chosen to be transfected with the *trans*-replication system, namely BHK-21, U2OS, Huh7, COP-5 and compared to BSR T7/5 cells. A new set of *trans*-replication system constructs (replicase plasmid and template) were designed for expression in BSR T7/5 cells driven by the RNA polymerase of bacteriophage T7 (**I; Fig. 1A**). In the presence of P1234-GAA in which the polymerase is inactive, Fluc and Gluc were still detected at a background level. Hence, the fold change was calculated and analysed. Low levels of Fluc were detected with the CMV promoter in all cell lines but the T7-based system in BSR T7/5 cells showed high Fluc activity. High levels of Gluc activity was seen in all cell lines, implicating efficient subgenomic RNA synthesis (**I; Fig. 2**). Next, the T7 and CMV promoter systems were compared head-to-head based on protein expression and viral RNA synthesis in U2OS and BSR cells (**I; Fig. 3**). Western blot analysis revealed that U2OS produced the least amount nsPs compared to BSR T7/5 cells (T7 and CMV systems). Conversely, RNA amplification was the highest in U2OS cells where the levels of genomic (full length template) to subgenomic RNA production was rather proportionate. Viral protein production was the highest under the T7 promoter in BSR T7/5 cells.

Synonymous codons have been shown to affect mRNA secondary structures, protein expression levels and protein folding[96]. Codon usage varies be-

tween organisms and since CHIKV replicates in both vertebrates and mosquitoes, a compromise has to be reached. In the case of CHIKV *trans*-replication systems, using fully codon optimised replicase plasmids increased their performance in a mammalian cell line. It is worth noting that the genomic RNA can be synthesised by the T7 RNA polymerase or cellular RNA polymerase II, thus explaining why it was also detected in the presence of the inactive replicase P1234-GAA. A background level was always observed for Fluc activity and for Gluc to a lesser extent. Hence, it is important to assess the efficiency of the *trans*-replication system (P1234 and mutants) based on the fold change. In previous studies, BSR T7/5 cells have been the 'instrument' of choice for *trans*-replication systems but a critical limitation is that the constructs have to be T7-driven. Hence, it was of relevance to investigate viral replication in other cell lines and using other promoter systems. We have shown that the CHIKV *trans*-replication system is versatile and can be used in various cell lines albeit the effectiveness might not be always optimal. This also indicates that the efficiency of the *trans*-replication system is host-specific (**publication I**). We observed that even when a small amount of replicase proteins were generated, they could be extremely active in RNA replication.

When comparing the T7 and CMV promoters, two features should be taken into account: replicase proteins production and template RNA amplification. NsPs expressed from the replicase plasmids under the T7 promoter and Fluc activity were at the highest level in BSR T/5 cells. BHK-21 and U2OS were the most efficient cell lines for the CMV-driven templates. Based on this information, further characterisation of the CHIKV *trans*-replication systems was done using BSR T7/5 for T7-driven constructs whereas U2OS cells were used for the CMV-driven constructs.

4.1.1 Successful tagging of CHIKV replicase proteins

Four positions were chosen for the insertion of eGFP (denoted as ^{E})at the C-terminus of nsP1 (P1^{E}234-A, P1^{E}234-B P1^{E}234-C P1^{E}234-D), two positions in the middle of nsP2 (P12^{E}34-A, P12^{E}34-B), only one position in nsP3 (P12 3^{E}4) and nsP4 was tagged at the C-terminus with eGFP but also combinations of smaller immunological tags (P1234^{E}, P1234^{SF}, P1234^{HS}, P12 34^{HF}) (**I; Fig 4**). Following transfection of each of these replicase plasmids with the Fluc-Gluc template, the tags were detected by Western blot thus ensuring that proper expression of the fusion proteins occurred. As predicted, a minimal effect was observed on the replication activity when nsP3 was tagged. The same was seen for

P12^{E}34-A. The transcription and translation activity of P12^{E}34-B was seen to be reduced by 100-fold. The tags in P1^{E}234-C and P1234^{SF} had a milder effect on replication in comparison to the other constructs with modified nsP1 or nsP4.

Viral genomes are compact and often reject insertions, such as markers added to the viral genome, or revert mutations in subsequent passages. This aspect can be problematic for the investigation of replication and virus-host interactions. *Trans*-replication systems depict a different story as viral particles are not produced. CHIKV replicase proteins are known to be intolerant to modifications, especially at their N-termini. Uncoupling protein expression and RNA template replication solves some of these problems as has previously been demonstrated with SFV and Sindbis *trans*-replication systems[49-50, 56, 67]. However, insertions in critical areas affect the stability of the protein. Previous studies involving SFV or CHIKV have reported that nsP2 and nsP3 can accommodate tags[10] but nsP1 and nsP4 are known to be less tolerant[20]. However, in the case of P12^{E}34-B, the marker was inserted in the protease region of nsP2 and thus, it is possible that the polyprotein processing was adversely affected accounting for the fold reduction observed. nsP1 and nsP4 being the most problematic, it was not surprising that replication was severely hampered by the insertion of eGFP. However, our results show that the C-terminus of nsP1 is suitable for the introduction of eGFP while nsP4 can accommodate some smaller markers such as HF without annihilating replication.

4.1.2 From *trans*-replication systems to replicon vectors and viruses

Based on the information obtained from the attempts to tag the replicase proteins in the *trans*-replication system, CHIKV replicon vectors were designed and their *in vitro* transcribed RNAs were transfected in BHK-21 cells. Repl-1^{E}, Repl-2^{E}, Repl-3^{E}, Repl-4^{SF} and Repl-4^{HF} each harboured the tag in the position that exhibited the highest replication activity in the *trans*-replication system and was compared to Repl-wt (**I; Fig 5A**). All these constructs, except Repl-4^{HF}, displayed significant cytotoxicity, suggesting competent viral RNA replication. Furthermore, replicase protein expression was high and equivalent to the *trans*-replication system, again with the exception of Repl-4^{HF}. The SF and HF tags in nsP4 gave very different results in replicons which led to their investigation in the context of infectious genomes (**I; Fig 5C**). A comparison of recombinant viruses ICRES-wt, ICRES-4^{SF} and ICRES-4^{HF} revealed that although their level of protein expression was similar, the infectivity of the tagged versions was lower, with ICRES-4^{HF} being the lowest. Additionally, further analysis revealed that ICRES-

4^{HF} had eventually lost the tag probably due to genetic instability. In replicon experiments, Repl-4^{HF} showed poor expression of nsP2 and nsP4 accounting for the instability caused by tagging the C-terminus of nsP4 with the HF combination tag.

4.1.3 Subcellular localisation of replicase proteins using CHIKV replicons and a *trans*-replication system in U2OS cells

Using tagged CHIKV replicon vectors and *trans*-replication constructs, it is possible to localise the replicase proteins in cells. The template T7-Rluc-Tom was used in which the Tomato marker is expressed from the second ORF under the control of CHIKV subgenomic promoter (**I; Fig 1B** – last two constructs). Hence, only cells displaying red fluorescence were analysed. Replicons and *trans*-replication systems presented the same features: nsP1 was found primarily on the plasma membrane, nsP2 mostly in the nucleus, nsP3 was seen strongly clustered in the cytoplasm while nsP4 was scattered throughout the cytoplasm.

Notably, all the cells exhibiting red fluorescence harboured active viral replication and, for the *trans*-replication system, the transfection efficiency was about 20% (unpublished data). The aforementioned cellular locations of the nsPs correspond to the excess replicase proteins that are not part of the replication complex and are probably interacting with host factors. Furthermore, the *trans*-replication system is, to some extent, an 'artificial' system and nsPs are continuously being generated. Similar subcellular localisations of nsPs outside of the replication complex had previously been observed for untagged infectious CHIKV viruses[118]. Detection of replicase complexes is impossible at this resolution. Moreover, it is probable that each replication complex contains a low amount of nsPs, which may not be enough for detection by fluorescence microscopy. The important indicator of viral replication, the intermediate dsRNA, was detected primarily on the plasma membrane for all tagged replicon and *trans*-replication constructs. Since all nsPs come from a polyprotein, one would expect that they exist in the cell in equal numbers but a discrepancy was observed in the intensity of eGFP and anti-Flag conjugated antibodies. For example, nsP3 was always easily identified, perhaps due to the aggregation. However, nsP4 often displayed much weaker signals probably because of its targeted degradation.

4.1.4 Spherule formation in CHIKV using *trans*-replication systems

Abundant spherules were detected in tagged and untagged CHIKV *trans*-replication systems (**I; Fig 7**). They were single-membraned, adjoined to the

plasma membrane, pouch-like and with a neck structure opening towards the cytoplasm. An electron-dense dot-like structure can be seen in the middle and is believed to be condensed nucleic acid. The spherules detected with the CHIKV *trans*-replication system were similar to those observed in previous studies with SFV[50, 131]. These invaginations are the site of active viral RNA synthesis and are very rarely seen in the presence of the inactive polymerase expressed from P1234-GAA (T7 and CMV). Furthermore, spherule formation was not affected by the presence of the tags in CHIKV replicases.

4.1.5 Requirements for CHIKV nsP2 protease activity

The protease region of nsP2 was fused to a histidine tag (His) at the N-terminus and purified as an active recombinant protein. Mutated versions were also made including C478A (Cysteine 478 residue of CHIKV nsP2 to Alanine), S482A (Serine 482 residue to Alanine), the combination (C478A + S482A) and W479A (Tryptophan 479 residue to Alanine). These mutant proteins were able to fold similarly to His-nsP2 (wildtype), with no detectable structural deformity, except for the W479A mutant that showed abnormally high ellipticity (**II; Fig 2b & c**). C478A and W479A hampered the ability of nsP2 to cleave 1/2 and 3/4 sites (**II; Fig 5**). The mutations were transferred to SFV and CHIKV *trans*-replication systems, under the T7 promoter, for transfection in BSR T/5 cells. Tmed and T7_Rluc_Tom were used for with the SFV and CHIKV replicase plasmids respectively. Northern blot experiments revealed that genomic and subgenomic viral RNA synthesis was abolished for the mutants with the exception of the S482A mutant (**II; Fig 6**). S482A produced slightly more minus-strands than the control P1234. To confirm the ability of S482A to replicate, BHK-21 cells were infected with CHIKV harbouring the S482A mutation at MOI 10 (**II; Fig 8**). Numerous spherules were observed at the plasma membrane as well as internalised CPVs containing spherules.

Previous studies have shown that the nsP2 of alphaviruses acts similarly to papain-like proteases[41]. We observed that the substrate requirements of CHIKV nsP2 protease were similar to those of SFV nsP2. Contrary to a previous study[115], the Ser482 residue in the active site of CHIKV nsP2 does not compensate for the loss of the Cys478 residue. For different alphaviruses the corresponding position can be occupied also by Cys or Thr residues indicating that some variation is tolerated at position 482. The protease region of nsP2 in alphaviruses is very conserved. Hence, it can be hypothesised that an antiviral inhibiting the protease activity of one alphavirus will most likely inhibit the others as well. The

role of the Cys478 residue in the catalytic site of nsP2 protease was found to be critical for polyprotein processing and viral replication. Moreover, the C478A mutant blocked all the essential biological activities of the corresponding replicases and viruses. The strange folding capabilities observed with W479A recombinant protein might be the result of enzymatically inactive aggregates.

4.1.6 RNA replication and cytotoxicity

Replicase constructs harbouring mutations thought to impact the cytotoxic effect of nsP2 were designed for analysis in a *trans*-replication system with the template CMV-Fluc-Gluc, in U2OS cells (**I; Fig 8A**). These mutations were Pro718 to Gly (PG), Lys192 to Asn (KN), Glu117 to Lys (EK), the insertion of five amino acids after residue 647 of nsP2 (5A), as well as combinations such as EKPG and 5APG. Western blot analysis (**I; Fig 8B & 8C**) revealed that KN, PG and EKPG enhanced the production of nsPs whereas EK had no effect on replicase protein expression. The 5A and PG mutations on their own resulted in a slight increase in nsP2 compared to P1234 but did not affect replication. However, the combination 5A+PG did not have an effect on nsP2 expression and Gluc activity was hindered. A significant reduction in Gluc activity was also observed from the EK, EKPG while the KN mutation was lethal for viral replication (**I; Fig 8C**).

nsP2 is known for causing a strong cytopathic effect and, causes transcriptional and translational shutdown in cells[35]. Previous studies with SFV have demonstrated that 5APG and EKPG reduce cytotoxicity[135]. It had also been shown that viruses harbouring EK and EKPG were too unstable to allow any kind of investigation of functionality[140] but with the *trans*-replication system, it was possible to observe the decrease in subgenomic RNA production with EK and EKPG. It is interesting that even though the KN mutation provided a boost in nsP expression, the change was not reflected in viral replication. This indicates that high protein expression levels do not automatically result in more efficient viral replication. This observation could be further confirmed by examining the effects of EK and EKPG. EK clearly hampered viral replication and although PG boosted protein expression, it did not compensate for the loss of viral replication activity. It is possible that KN affects NTPase, helicase and/or RNA triphosphatase activities which are vital for CHIKV replicase function. While 5A and PG mutations on their own did not affect subgenomic RNA synthesis, together they had a negative effect on viral replication. Therefore, I hypothesise that 5APG gives rise to a version of nsP2 in which folding is affected, possibly

hindering the protease catalytic domain (there is evidence that 5APG reduces the rate of polyprotein processing[140]), or renders nsP2 less efficient in participating in viral replication complex formation thus impeding the viral RNA synthesis.

4.2 FHV *trans*-replication system; uncoupling viral protein expression and RNA Synthesis

As mentioned in the introduction, FHV is one of the simplest viruses known as only protein A and the RNA template are required for viral replication. The design of an FHV specific *trans*-replication system was established under the control of the T7 promoter for use in BSR T7/5 cells. The replicase plasmid P_HA corresponds to FHV RNA1 with the addition of an HA tag at the C-terminus of protein A, less the 5' and 3' UTRs (**III, Fig 1b**). P_GAA contains a double missense mutation in the catalytic domain of the polymerase. The template plasmid T_Rluc contains the UTRs, includes a frameshift that prevents the expression of protein A and Rluc marker inserted under the control of the subgenomic promoter, in fusion with the B2 open reading frame. Following transfection in BSR T7/5 cells, protein A was expressed from P_HA and recruited the RNA template generated from the T_Rluc construct. This resulted in high levels of Rluc detected in BSR T7/5 cells and extremely high viral RNA synthesis, thus demonstrating the efficiency of FHV *trans*-replication system (**III, Fig 1d & 1e**). The GAA mutation did not hinder protein expression and did not yield any RNA transcript.

BSR T7/5 cells had been formerly used for SFV and CHIKV *trans*-replication systems (**I, II**) and had proven to yield high levels of RNA transcripts. It was earlier mentioned that, in the case of CHIKV T7-driven P1234-GAA, genomic RNA was detected in BSR T7/5 cells at very low levels. Similar, to these previous studies, mRNAs of template plasmids can be transcribed intracellularly by the T7 polymerase thus accounting for the background levels of Rluc detected with P_GAA + T_Rluc and T_Rluc transfected alone. Interestingly, these aforesaid combinations did not produce genomic or subgenomic viral RNA at detectable levels. Therefore, it seems that the T7 RNA polymerase produces less mRNA transcripts (background) in the FHV *trans*-replication system than in the CHIKV *trans*-replication system but these transcripts are replicated very efficiently in the presence of the active FHV polymerase.

4.2.1 Mitochondria act as replication niches for FHV replication

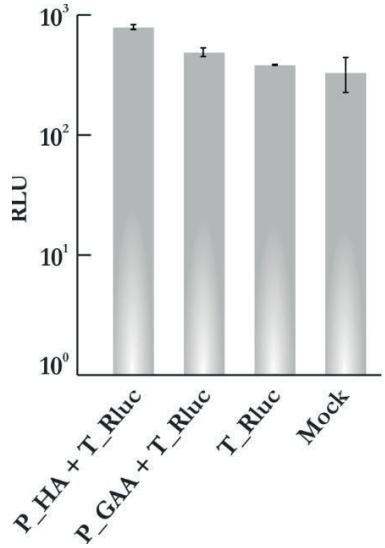
Subcellular localisation of protein A was studied using the template T_eGFP that expresses green fluorescence upon activation of the subgenomic promoter (**III; Fig 2a**). Using antibodies to detect the HA tag and Tom-20 (mitochondrial import receptor subunit TOM20 homolog), protein A was found to be strictly associated with mitochondria as expected. Moreover, the presence of dsRNA was indicative that mitochondria was the location of active viral RNA synthesis. P_GAA was also localised in mitochondria, due to the mitochondrial membrane anchor domain within protein A. However, no dsRNA was detected for P_GAA.

Spherules generated by the FHV *trans*-replication system arose from outer-mitochondrial membranes in cells harbouring active viral replication (**III; Fig 2b**). All the mitochondria in each replicating cell exhibited this phenotype. The morphology of mitochondria was severely altered; the intermembrane space appeared swollen to make space for the spherule and the cristae were barely visible. Considering the late timepoint chosen after transfection, it is not surprising that no intermediate structures were seen. A previous study by Kopek *et al* described a 'zippering' effect of mitochondria following transfection in *Drosophila* cells with protein A alone. In their experiment, the outer membranes of mitochondria tightly connected and clustered with projections interlocking like a zipper[64]. This effect was not observed in our experiments. Finally, there were no spherules when P_HA alone was transfected.

4.2.2 Temperature is a key factor for FHV replication in cells and *in vitro*

Several methods were attempted for the isolation of mitochondria following transfection of P_HA + T_Rluc in BSR T7/5 cells (unpublished data). Ultimately, a differential centrifugation protocol based on Frezza *et al* [32] produced a crude mitochondrial pellet (CMP) of suitable purity and was chosen for sample preparation. The CMP was used in an *in vitro* replication assay (IVRA) previously described to be efficient for FHV replication[123]. High synthesis of both genomic and SgRNA was observed after an incubation of 90 minutes at 30°C (**III; Fig 3a**). Further characterisation of the FHV IVRA showed that the first transcripts were detected within the first 30 minutes of the reaction (**III; Fig 3b**). To investigate the effect of temperature on RNA synthesis, the IVRA was performed at 25°C, 30°C and 37°C (**III; Fig 3c**). Expectedly, fewer RNA transcripts were detected at 25°C. However, a substantial increase in RNA synthesis was observed

Figure 5: FHV *trans*-replication at 37°C. BSR T7/5 cells were transfected with the indicated plasmids and luciferase activity was measured after 40 hours. The cells were kept at 37°C at all times. No significant difference was seen between P_HA + T_Rluc and the background levels.



at 37°C. Nevertheless, the other IVRA experiments were performed at 30°C unless stated otherwise. The ability of protein A to remain active at 37°C *in vitro* challenges results obtained in BSR T7/5 cells as shown in Figure 5 and Figure 6. When P_HA + T_Rluc was transfected into BSR T7/5 cells followed by incubation at 37°C, the luciferase signal obtained did not go much beyond background levels (Figure 5) although protein A was expressed (Figure 6).

Since FHV's primary hosts are insects, the preferred temperature for experiments in BSR T7/5 cells was 28°C. The fact that protein A was inactive when expressed in cells at 37°C suggested that an important replication step is temperature sensitive. Therefore, the increase in RNA synthesis at 37°C *in vitro* was very surprising. It could be that the assembly of the FHV's replication complex is a sensitive process requiring the temperature to be 28°C but, once formed, can perform at higher temperatures. Alternatively, mitochondria might be operating at different temperatures than the rest of the cell and enzymes taking part in the respiratory chain function maximally at about 50°C[17]. Chrétien *et al* stated that

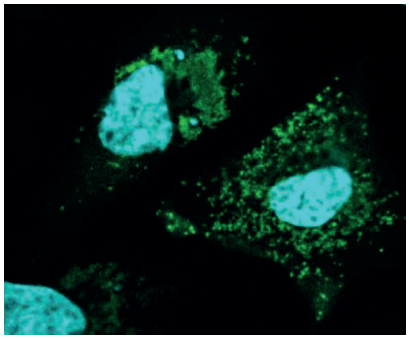


Figure 6: Protein A expression at 37°C. BSR T7/5 cells were transfected with P_HA and incubated for 18 hours at 37°C. Protein A was detected using α-HA antibodies, seen in green. Nuclei are shown in cyan.

the temperature of mitochondria (at least in HEK293 cells) is regulated by the rate of respiratory electron flux. Considering the substantial morphological changes mitochondria undergo during FHV replication, it can be assumed that the respiratory chain is affected. Moreover, the CMP isolation procedure might exacerbate those changes thus, explaining why this disparity in the temperature phenotype was observed.

4.2.3 FHV replicates only the endogenous RNA template and synthesises plus-strands *in vitro*

We attempted to resolve whether an already-formed FHV replication complex can recruit an exogenous RNA template. A new template, FHV_T was designed by deleting the Renilla luciferase marker from T_Rluc, thus making it shorter. The new combination P_HA+FHV_T was transfected in BSR T7/5 cells, the CMP was isolated and RNA synthesis was observed under IVRA conditions (**III; Fig 3e**). When an *in vitro* transcribed T_Rluc RNA (capped or uncapped) was added prior to the IVRA, genomic and SgRNA was observed again with sizes corresponding to FHV_T but not T_Rluc. This showed that the CMP was unable to replicate an externally provided RNA template. A CMP preparation containing only protein A showed no activity in the IVRA.

The polarity of the RNA products generated in the IVRA was determined based on their binding affinity to radioactively labelled capture probes specific to either the plus-strand or the minus-strand. RNA synthesised *in vitro* from P_HA+T_Rluc CMP strongly hybridised with the plus-strand probe and insignificantly (similar to the background) hybridised to the minus-strand probe.

An IVRA specific for FHV was previously described by Short *et al*[123]. In their experiments, they claimed that: 1) CMP isolated from infected *Drosophila* cells was poorly reactivated *in vitro*, and 2) providing an exogenous RNA template to the IVRA boosted replication. Contrary to these claims, we observed a robust reactivation of viral replication with the endogenous RNA *in vitro*. These differences could be the result of hosts specific mechanisms: *Drosophila* cells are fundamentally different from BSR T7/5 cells. Moreover, Short *et al* used wildtype viruses as opposed to the *trans*-replication system in our case. The question of whether an already formed viral replication complex can recruit another RNA template has been attempted for SFV but not resolved[57, 101]. Even though we clearly demonstrated that the CMP cannot replicate an exogenous template, it does not exclude the possibility that this RNA could still be recruited by the rep-

lication complex. The IVRA generated predominantly RNA transcripts of positive sense. Formation of spherules is believed to occur upon minus-strand synthesis. Since the mitochondria in the CMP are most likely already saturated with spherules, presumably no new spherules were formed in the IVRA. Moreover, protein A present in the spherules in the CMP was already interacting with a minus-strand and therefore there was no need for the generation of new minus-strands. Another explanation, although doubtful, could be that the CMP lacked some unknown host factors necessary for the production of the minus-strand. Ertel *et al* suggested that the crown structure made at the neck of FHV spherules is formed first and RNA is then recruited to the membrane[29]. While we have not investigated spherule formation following CMP isolation and IVRA, it is probably not the case. In the IVRA, adding *in vitro* transcribed T_Rluc to a CMP preparation transfected with only P_HA did not yield any RNA transcripts. Therefore, this suggests that the introduction of the RNA template at a later time point affects viral replication.

4.2.4 FHV capping domain and replicase protein mutants

Four of the most conserved aa residues in the putative capping domain of protein A (N-terminus) were mutated in an attempt to investigate the role of the capping activity in viral replication. The residues H93, R100, D141 and W215 were mutated to Ala. The P_GAA (inactivated polymerase mutant) and Δ2-35 (membrane-binding domain deletion) were taken as controls. The replicase mutants were transfected in BSR T7/5 cells together with T_Rluc. Even though the mutations did not affect protein expression (**III; Fig 4b**), viral replication was abolished for all the mutants except for the W215A mutant. W215A showed some activity but the luciferase signal was markedly reduced compared to P_HA (**III; Fig 4a**). Active viral RNA synthesis both in cells and *in vitro* (**III; Fig 4c & d**) was not detected for all replicase mutants with the exception of W215A which showed faint bands corresponding to the genomic and SgRNA. In an attempt to rescue viral replication, the replicase mutants were transfected in BSR T7/5 cells together with an *in vitro* transcribed T_Rluc RNA. Again, W215A was the only replicase mutant capable of replication (**III; Fig 5**).

W215 corresponds to an important, highly conserved tyrosine residue in alphaviruses[3-4]. It appears that W215 plays a lesser role in replication compared to the other residues. This is in contrast to what was observed in earlier studies involving SFV and Brome mosaic virus (BMV), where mutating the correspond-

ing tyrosine residue abolished replication[1, 57]. Thus, there might be some mechanistic differences in RNA synthesis and RNA capping between these two virus groups. H93 has been suggested as the covalent guanylate site involved in RNA capping[4], R100 is a highly conserved charged residue[3] and D141 residue is thought to bind S-adenosylmethionine[4]. Interestingly, mutating these three residues prevented RNA synthesis completely and the effect cannot be rescued by providing an already capped RNA. It could be that these residues in the putative capping domain of FHV are also involved in the recruitment of the viral RNA during replication complex formation.

4.2.5 A comparison of SFV and FHV *trans*-replication systems

The features of the SFV *trans*-replication system have been described in previous studies[51, 56, 131]. Here, we used P123HA4 which produces nsPs 1-4 and nsP3 is fused with an HA tag. The template T_med consisted of Rluc under the T7 promoter and a Tomato fluorescent marker under the subgenomic promoter. CFP_Stluc consisted of a cyan fluorescent protein under the control of the T7 promoter and Rluc under the subgenomic promoter. P123HA4-GAA was used as a control since it contains a mutation in the catalytic domain of the SFV polymerase in nsP4 (**III; Fig 6a**). Both the SFV (P123HA4+Tmed and P123HA4+CFP_Stluc) and the FHV (P_HA+T_Rluc) *trans*-replication systems were transfected in BSR T7/5 cells. The SFV system was incubated at 37°C whereas the FHV system was incubated at 28°C. Luciferase signals measured after 16 hours and 40 hours respectively were very high, well above the background levels obtained with the GAA mutant (**III; Fig 6b**). When the same combinations were tested in Northern blot using a probe that recognises the Rluc sequence (**III; Fig 6c**), active RNA synthesis was observed in all combinations that included an active replicase. Based on their replication and viral RNA synthesis levels and compared to their inactivated polymerase counterpart, the FHV *trans*-replication system appears to be more efficient than the SFV *trans*-replication system.

To test whether SFV and FHV can replicate in the same cell, BSR T7/5 cells were transfected with the SFV combination P123HA4+Tmed and the FHV P_HA+T_eGFP altogether. The cells were kept at 28°C (not an optimal temperature for SFV) and analysed by immunofluorescence after 40 hours. Three phenotypes were observed; green from eGFP (FHV replication only), red from the tomato marker (SFV replication only) and in some cells both signals were seen

(**III; Fig 6d**). CLEM experiments were performed to verify whether the two viruses could form their spherules in the same cell. Only the cells expressing both red and green immunofluorescence signals were analysed. Unfortunately, only SFV spherules were seen at the plasma membrane whereas the mitochondria looked normal. The SFV (red) immunofluorescence signal was strong showing that the *trans*-replication system worked quite well even under suboptimal conditions. This experiment had a few obstacles. First, the transfection efficiency was very low. In fact, the transfection efficiency of the T7-driven CHIKV *trans*-replication system was found to be about 18 % when only two plasmids (unpublished data) were transfected. Hence this ratio is expected to be much lower when transfecting four plasmids. Secondly, it is possible that even though all plasmids are introduced in the same cell, the replicase proteins are not able to find and recruit their corresponding template. Although we did not quantify the immunofluorescence signals, we observed that, in cells where both signals were seen, the green to red ratio was disproportionate. For example, the green signal detected from cells replicating both systems was much weaker than in cells were only FHV was replicating. Likewise, for SFV, cells replicating only SFV *trans*-replication system displayed a much stronger red phenotype compared to those where both FHV and SFV were replicating. Both systems are competing for the same T7 polymerase so it would be interesting to test whether this observation would arise if they would use different promoter systems. Both systems could also be competing for the same host factors. If this were the case, it would indicate that SFV and FHV use similar replication strategies even though the membrane requirement is different. It was unexpected that the CLEM experiment did not show FHV spherules as the green signal arises from SgRNA synthesis, which is the hallmark of viral replication. A possible explanation would be that usually only one system could establish a high-level replication in an individual cell.

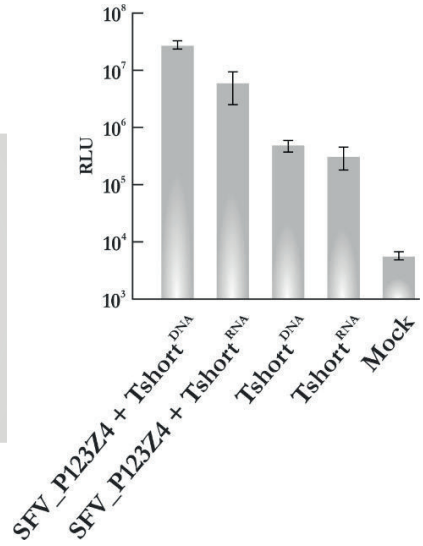


Figure 7: Alphavirus *trans*-replication with combined DNA and RNA transfection. BSR T7/5 cells were transfected with the combinations shown. P123Z4 was transfected as DNA and Tshort as DNA or RNA. Luciferase activity was measured after 16 hours.

Interestingly, transfection of the replicase plasmid in DNA form and the template as an *in vitro* transcribed capped RNA resulted in active replication almost as robust as when both are transfected as DNA (**III; Fig 5**). Hence a similar experiment was done with SFV *trans*-replication system in BSR T7/5 cells as shown in Figure 7. Here again, transfection of the DNA+RNA combination worked unexpectedly well. The replicase plasmid, being in DNA form upon transfection, would have to undergo transcription and translation. Thus, we thought that the RNA template would have been already degraded by the time the replicase proteins are translated and available for recruitment. The fact that this unusual combination worked so well further testifies to the adaptability of *trans*-replication systems.

CONCLUDING REMARKS

The main focus of the study was to investigate the interactions between the viral replicases and the RNA genome as well as spherule formation for CHIKV and FHV by means of *trans*-replication systems. Additionally, SFV and FHV *trans*-replication systems were compared.

The main findings and further questions include:

I. The CHIKV *trans*-replication system mimics viral replication and can be adapted for use in various cell lines. However, the efficiency is host-specific. The CHIKV *trans*-replication system allowed the visualisation of nsP1 on the plasma membrane, nsP2 in the nucleus, nsP3 in cytoplasmic aggregates, nsP4 scattered in the cytoplasm and spherules at the plasma membrane. Next, it would be interesting to investigate the functions of the C-terminus of nsP1 and nsP4 by mutagenesis using the *trans*-replication system.

II. Cys478 and Trp479 residues are crucial for the protease activity of CHIKV nsP2 while the substitution of Ser482 to Ala has a negligible effect. In the future, it would be interesting to know what's happening in the interplay of $P12^{5A}34$ and $P12^{PG}34$, is nsp2 being degraded instead of rpb1? Is the flexibility of the protease domain reduced? Is the catalytic rate more active?

III. The FHV *trans*-replication system revealed abundant spherules associated with the outer membrane of mitochondria. FHV protein A is inactive in BSR T7/5 cells but active *in vitro* at 37°C. Only the endogenous template was replicated *in vitro* and the RNA transcripts generated were of positive polarity. Mutating the conserved residues H93, R100 and D141 abolished viral replication which could not be rescued by providing an already capped RNA genome. The elucidation of the specific mechanisms of the FHV capping domain and its influence on viral replication will open new avenues in terms of antivirals because of its similarities with alphaviruses and capping enzymes are promising antiviral targets.

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